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Original Research Article

Long-Term Effects of Low-Dose Chlorpyrifos Exposure on Serum Albumin Levels in Male Wistar Rats

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Abstract

Background: Chlorpyrifos is one of the organophosphate pesticide types frequently utilized as a pest control agent in Indonesia. Despite its effectiveness in combating pests, the residue levels of chlorpyrifos in the environment and plants have raised serious concerns. Long-term accumulation of chlorpyrifos in the body can lead to organ damage, particularly in the liver and kidneys, which may decrease serum albumin levels.

Objective: To investigate the impact of low-dose chlorpyrifos exposure over time on serum albumin levels in Wistar rats.

Methods: This study used a posttest-only randomized control group design method, conducted from May until September 2023. Thirty male Wistar rats were divided into five groups: the normal control group (Kn) received normal saline solution (+5% Tween 20) orally for 56 days, while the treatment groups (K1, K2, K3, and K4) were administered chlorpyrifos at a dose of 5 mg/kg body weight for 7 days, 14 days, 28 days, and 56 days orally. Serum albumin levels were measured using the dye-binding method with a spectrophotometer.

Results: The measurement results indicate that the normal control group (Kn) had the highest serum albumin levels (4.326±0.519 g/dL). Serum albumin levels decreased in the groups treated with chlorpyrifos. The longer the chlorpyrifos exposure, the lower the serum albumin levels. The lowest serum albumin levels were found in group K4 with chlorpyrifos exposure for 56 days (2.826±0.358 g/dL). Statistical analysis using One-way ANOVA and Post Hoc LSD tests showed significant differences ($p<0.05$) between all treatment groups (K1, K2, K3, and K4) and the control group (Kn).

Conclusion: This study shows that administering low-dose chlorpyrifos over a period of 7 to 56 days has a significant effect in reducing serum albumin levels in Wistar rats. The clinical implications of this decrease in serum albumin levels need to be considered in the context of exposure to organophosphate pesticide residues in humans.

Keywords: *Organophosphates; hepatic toxicity; proteins; pesticide residues.*
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INTRODUCTION

Organophosphates are the most widely used type of pesticide in the world due to their effectiveness in eradicating agricultural pests.[1] One of the organophosphate pesticides frequently used in Indonesia is chlorpyrifos.[2] Chlorpyrifos accounts for 40% of the total organophosphate pesticides used.[3] The effectiveness of chlorpyrifos in pest eradication is proportional to the residue it generates in the environment and plants. A study revealed that using chlorpyrifos in agriculture leads to chlorpyrifos residues in soil exceeding the maximum pesticide residue.[2] Long-term residue exposure can lead to the accumulation of chlorpyrifos in the body, resulting in symptoms of chlorpyrifos toxicity.[4]

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Long-term accumulation of chlorpyrifos can damage various organs, including the nervous, cardiovascular, respiratory, liver, and kidneys.[4] Studies indicate that administration of 1 mg/kg BW of sub-chronic chlorpyrifos to experimental animals for 90 days damaged hepatocytes.[5,6] The metabolic process of chlorpyrifos by esterase and cytochrome P450 enzymes in the liver produces more toxic compounds, namely chlorpyrifos-oxon, and 3,4,5-trichloro-2-pyridinol causing oxidative stress and triggering liver cell damage.[7,8] Liver cell damage also results in decreased albumin production, causing hypoalbuminemia.[5] Additionally, the effects of long-term accumulation of chlorpyrifos in the body can also cause kidney damage.[7] Chlorpyrifos is excreted from the body through the kidneys in the form of 3,5,6-trichloro-2-pyridinol (TCP), a metabolite that is more soluble in air and can be easily excreted by the kidneys into the urine.[9] Accumulation of TCP in the kidneys can cause oxidative stress on glomerular epithelial cells, triggering damage to epithelial cells in the glomerulus.[7] Damage to the glomerular epithelium can interfere with the ability to filter substances visually so that albumin and other proteins can leak into the urine and cause hypoalbuminemia.[10]

Hypoalbuminemia can impact various bodily functions, including systemic edema and pleural effusion, due to decreased oncotic pressure in the blood vessels. Hypoalbuminemia can also interfere with transporting various hydrophobic molecules, such as lipid-derived hormones (thyroid, estrogen, testosterone, and cortisol), lipid-based drugs, and other air-insoluble ions.[11] Hypoalbuminemia also impacts decreased cell regeneration ability, reduced immunity, and impaired neutralization of free radicals.[12]

The results of research on the effect of chlorpyrifos on albumin levels in rats are still limited[13], more often carried out on fish as experimental animals.[14–16] Those studies showed decreased albumin levels in the group given chlorpyrifos. In a study conducted by Ravikumar et al. (2020), Wistar rats were given chlorpyrifos at a high dose of 25 mg/kg BW for 28 days, and albumin levels were checked on the 15th and 29th days. The study results showed a significant decrease ($p<0.05$) in total protein and albumin levels in the group given chlorpyrifos on the 15th and 29th days of the experiment.[13] Studies on organophosphate poisoning patients showed symptoms of hypoalbuminemia in those poisoned by chlorpyrifos.[8]

Studies in mammalian animals have focused more on liver and kidney cell damage caused by chlorpyrifos, and the results have had different effects on these organs. In addition, these studies have not provided much data on the decrease in albumin levels caused by chlorpyrifos. Administration of a low dose of chlorpyrifos (2 mg/kg BW) for 12 hours in rats causes an oxidative stress response in the liver.[17] There were no changes in liver histology in rats at low doses (1 mg/kg BW) for 12 weeks.[6] High doses of chlorpyrifos (50 mg/kg BW) for 4 weeks showed proliferation of Kupffer cells in the liver and local bleeding in the kidneys.[7]

Research on administering low doses of chlorpyrifos over various periods in the same study is still limited. In *Esisenia fetida*, an earthworm species, chlorpyrifos toxicity was lower after 28 days compared to groups exposed to other compounds but higher after 56 days of administration.[18]

Therefore, using a time-series study design, research on albumin in long-term low-dose chlorpyrifos administration can help identify possible impacts over a more extended period. By monitoring albumin levels over time, it can be seen whether low-dose chlorpyrifos administration can cause significant changes in albumin levels. This study is expected to provide new insights into the potential side effects of chlorpyrifos on the systemic body, especially in the context of albumin levels.

MATERIALS AND METHODS

Research Design and Experimental Animal

This research was conducted after obtaining ethical approval from the Health Research Ethics Commission of the Faculty of Medicine, University of Jember, with approval number 1813/H25.1.11/KE/2023. The research used a posttest-only randomized control group design method. A total of 30 healthy white male rats of the Wistar strain (*Rattus norvegicus*) aged 2-3 months, weighing 150-200 g, were randomly divided based on weight into five groups, with six rats each. The number of samples for each group was calculated using the resource equation method and correction factor to anticipate dropout. The five groups were the normal control group given 5 mL/kg BW saline (+5% Tween 20) orally for 56 days (K), the chlorpyrifos group 5 mg/kg BW orally for 7 days (P1), 14 days (P2), 28 days (P3), and 56 days (P4). The animals were kept in full hygienic conditions and had free access to fresh water and standard pellets. This study was conducted from May until September 2023 in the experimental animal house, Pharmacology Laboratory, and Clinical Pathology Laboratory Faculty of Medicine, University of Jember.

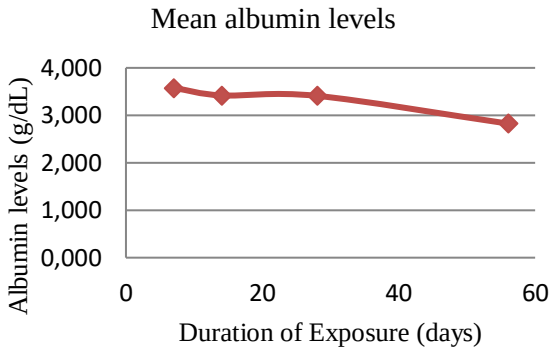

Figure 1. Graph of albumin levels according to duration of exposure

Table 1. Measurement of Serum Albumin Levels

Group	Mean Serum Albumin Levels (g/dL) (Mean±standard deviation)
Kn	4,326±0,519
K1 (7 days)	3,578±0,599
K2 (14 days)	3,42±0,704
K3 (28 days)	3,41±0,481
K4 (56 days)	2,826±0,358

Table 2. Results of Post Hoc LSD Test

	Kn	K1	K2	K3	K4
Kn		0,025*	0,008*	0,007*	0,000**
K1	0,025*		0,619	0,597	0,025*
K2	0,008*	0,619		0,975	0,071
K3	0,007*	0,597	0,975		0,076
K4	0,000**	0,025*	0,071	0,076	

Explanation: (*) indicates significant difference ($p<0.05$), (**) indicates highly significant difference ($p<0.001$)

Materials

This study used chlorpyrifos pestanal analytical standard (Sigma-Aldrich), saline, Tween 20, distilled water, reagen BCG Albumin (Dialab), and 30 male Wistar rats.

Preparation of Chlorpyrifos Solution

Chlorpyrifos solution 5 mg/kg BW (1/30 LD50) was made by weighing chlorpyrifos. After that, it was mixed with Tween 20, and then saline was added and stirred until evenly mixed.

Albumin Level Examination

After the treatment, the rats were terminated using intraperitoneal injection of pentobarbital at a dose of 200 mg/kg BW, and their blood was taken intracardially to examine serum albumin levels. Serum preparation began with incubation of rat blood for 10-20 minutes, then centrifugation was carried out at a speed of 3000 rpm for 10 minutes. The blood serum was separated from the solution and then put into micro tubes and labeled. Before serum albumin level examination, blank reagent and standard reagent were prepared by inserting 5 µL of distilled water and 500 µL of BCG reagent into the blank tube, and 5 µL of standard solution and 500 µL of BCG reagent into the standard tube. Serum albumin sample examination was done using the dye-binding method by inserting 5 µl of blood serum and 500 µl of BCG reagent into the sample tube.[19] All solutions were homogenized using a vortex and incubated for 10 minutes at 20-25 degrees Celsius. Albumin level readings were carried out by measuring the absorbance value of the solution using a spectrophotometer with a wavelength of 546 nm.

Statistical Analysis

The research data obtained were presented in tables and processed using One Way ANOVA statistical analysis with a confidence level of 95% ($\alpha = 0.05$), then continued with the Post Hoc LSD difference test.

RESULTS

The results of serum albumin levels measurement and the graph of albumin levels according to the duration of exposure are shown in Table 1 and Figure 1, respectively. Based on the average serum albumin levels measured in each group, it was found that the Kn group had the highest albumin levels compared to the other groups, at 4.326±0.519 g/dL. The lowest albumin levels were observed in group K4, which received chlorpyrifos for 56 days at 2.826±0.358 g/dL. The albumin levels from K1 to K4 showed a decreasing trend, indicating that with prolonged exposure to chlorpyrifos, the albumin levels in rats also decreased.

The obtained serum albumin level results were further subjected to comparative analysis using One-way ANOVA. The requirement for the one-way ANOVA test to be carried out is that the data must be normally distributed and homogeneous. Therefore, the data was previously analyzed using the Shapiro-Wilk normality test and the homogeneity test using the Levene test. The results of the normality and homogeneity tests of the data obtained $p>0.05$, which means that the data is normally distributed and homogeneous so that it can be continued with the one-way ANOVA test. Based on the One-way ANOVA test, a significance result of 0.002 ($p<0.05$) was obtained. The analysis was continued with the Post Hoc LSD test. The results of the Post Hoc LSD test (Table 2) showed a significant difference ($p<0.05$) in serum albumin levels between the control group (Kn) and the chlorpyrifos-exposed groups (K1, K2, K3, and K4), as well as between group K1 and K4. This difference can be attributed to the varying exposure durations, especially between K1 exposed for 7 days and K4 exposed for 56 days. The difference in exposure durations provides an insight into the accumulation of significant effects of chlorpyrifos exposure on serum albumin levels, thus demonstrating that with prolonged (chronic) exposure to chlorpyrifos, serum albumin levels will also decrease.

DISCUSSION

This study aims to prove the effect of low-dose chlorpyrifos administration on albumin levels in Wistar rats, using a time series, namely administration in 7, 14, 28 and 56 days. These times were chosen to represent acute (7 days), subacute (14 days), and subchronic (28 and 56 days) toxicity in animal toxicity assessment.[20] A low dose of chlorpyrifos was given to resemble the residual dose found in vegetables and fruits consumed by humans, considering that chlorpyrifos is often used in agriculture, especially to control pests in vegetable and fruit products.[21] Chlorpyrifos has an oral LD50 in rats ranging from 66-195 mg/kg BWor 150 mg/kg BW.[22,23] It is stated in research conducted by Noushi (2013) that a low dose is 1/30 of the LD50, so from the LD50, the dose range that can be used is 2.2 - 6.5 mg/kg BW or 5 mg/kg BW.[24]

Our study showed that the group given low-dose chlorpyrifos 5 mg/kg BW for 7, 14, 28, and 56 days had significantly lower serum albumin levels compared to the group not given chlorpyrifos. Normal albumin levels in male Wistar rats are 3-5.1 g/dL, and the group not given chlorpyrifos had albumin levels of 4.236 ± 0.519 g/dL, indicating that the levels were within the normal range.

In this study, it was found that the average albumin levels decreased in the group given chlorpyrifos, starting on the seventh day of treatment, namely 3.578±0.599 g/dL, and the lowest on the 56th day was 2.826±0.358 g/dL. A retrospective study by Noh *et al.* (2020) investigating the effects of organophosphate poisoning on 217 patients showed the presence of hypoalbuminemia. The study examined several organophosphates that were accidentally ingested, categorized as low dose (2 mg/kg body weight) and high dose (> 2 mg/kg body weight), followed by observation after 24 hours.[8] The doses are doses in humans, where the dose used in our study was a low dose after being converted to humans, which was 0.8 mg/kg BW (≤ 2 mg/kg BW). From Noh's study, organophosphates were shown to trigger an oxidative stress response in the liver, which can result in reduced albumin production.[8]

In this study, the exposure of low-dose chlorpyrifos was found to significantly decrease serum albumin levels, with an average of 3.41±0.481 g/dL at 28 days and a further decrease at 56 days with an average of 2.826±0.358 g/dL. This effect is believed to occur due to the accumulation effect of low doses of chlorpyrifos, causing increasingly severe hepatocyte damage resulting in decreased albumin production. In contrast, a study by Zhang *et al.* (2021) found that exposure to low-dose chlorpyrifos (1 mg/kg body weight) for 12 weeks did not show any significant changes in liver histological examination in rats.[6] This is because the dose exhibited significant hepatotoxicity in that study was 5.4 mg/kg body weight, leading to hepatotoxicity through changes in liver enzyme markers' profiles such as ALP, AST, LDH, and organ structure. Chlorpyrifos can elevate TNFα, IL-1β, and IL-6 levels and induce inflammatory responses with increasing doses.[6]

The metabolism of chlorpyrifos in the liver through hydrolysis by esterase enzymes and cytochrome P450 results in the formation of a more toxic compound called chlorpyrifos-oxon (CPO), which triggers liver cell damage through oxidative stress mechanisms. Liver cell damage affects the physiological function of the liver, one of which is the production of albumin protein. Research indicates that the decrease in serum albumin levels correlates with the degree of hepatocyte damage.[25] Such a condition can exacerbate the development of hypoalbuminemia resulting from chlorpyrifos exposure.[8]

Exposure to chemical substances such as chlorpyrifos can trigger oxidative stress in the liver, primarily due to the high production of reactive oxygen species (ROS), especially from mitochondria and NADPH oxidase. Elevated ROS levels can cause damage to liver cell components, including lipids, proteins, and nucleic acids. Additionally, oxidative stress can disrupt normal mitochondrial function, altering energy production processes within liver cells and leading to structural and functional changes in the liver that can affect overall organ performance. One consequence of this oxidative stress is the reduction in albumin production by the liver, leading to hypoalbuminemia. Therefore, oxidative stress plays a key role in the mechanism of liver damage and the decrease in albumin levels.[26,27]

Oxidative stress due to chlorpyrifos exposure leads to changes in metabolic function, ultimately resulting in cell death. The mechanism of non-cholinergic pro-oxidant effects on chlorpyrifos and chlorpyrifos-oxon (CPO) toxicity has been studied by Naime *et al.* (2020).[28] Exposure to chlorpyrifos and chlorpyrifos-oxon (CPO) results in a significant decrease in glutathione levels, preceding a significant reduction in cell viability. The study indicates that, apart from being a stronger acetylcholinesterase inhibitor, chlorpyrifos-oxon is also a potent pro-oxidant molecule. Thus, oxidative injury can contribute to liver and kidney damage as the human liver expresses CYP2B6, the main enzyme responsible for chlorpyrifos metabolism, into chlorpyrifos-oxon. Additionally, subacute exposure to low doses forms toxic oxon metabolites, causing hyalinization, vacuolization, nuclear necrosis, hepatocyte edema, and lipid degeneration. These morphological changes may also be associated with impaired cell function and lower antioxidant capacity.[28,29]

Apart from liver cell damage, hypoalbuminemia can also occur due to kidney damage. Research conducted by Sakinah et al. in 2024 showed that oral administration of low-dose chlorpyrifos pesticides in 7, 14, 28 and 56 days can cause increased BUN and creatinine levels and decreased glomerular diameter in the group given chlorpyrifos compared to the group without chlorpyrifos.[30] A study by Aung et al. (2022) investigated the exposure of chlorpyrifos to kidney damage in 18 rats treated with subacute and subchronic doses of 18 mg/kg body weight via subcutaneous injection. From the study, kidney damage was observed through oxidative stress processes. One of the most well-known mechanisms of organophosphate pesticide-induced kidney damage in subacute and subchronic exposure is through oxidative stress effects on the kidneys. Malondialdehyde (MDA) is a product of lipid peroxidation and is considered an oxidative marker. The results of the study indicated significant changes in oxidative damage as the basis of nephrotoxicity, as serum MDA levels increased and were strongly expressed in renal tubular cells in animals exposed to chlorpyrifos. [31]

Accumulation of 3,4,5-trichloro-2-pyridiol in the kidney can induce oxidative stress in glomerular epithelial cells, damaging the glomeruli's visceral epithelial cells (podocytes).[7] Damage or abnormalities in glomerular podocytes can disrupt their ability to filter substances selectively. As a result, albumin and other proteins may leak into the urine.[32] When the amount of albumin lost in the urine exceeds the liver's capacity to replace the loss, the level of albumin in the plasma decreases (hypoalbuminemia).[33]

One of the factors that can affect albumin levels is the age of the rats. In the study of Olukuran (2018), it was shown that there was a relationship between changes in urinary protein excretion in rats at various ages. The study proved that some of the molecular weight of protein in the urine of rats aged 1, 9, and 12 months was higher than that of rats aged 3 and 6 months. The total protein concentration in the urine of male and female rats aged 9 and 12 months was significantly higher than that of rats aged 1 and 3 months.[34] In our study, we used rats aged 2-3 months. Thus, selecting 2-3-month-old rats should not affect albumin levels in the rats we studied.

Our study has limitations: albumin levels were not checked at hour 0 to ensure that all groups had the same albumin levels before chlorpyrifos administration. However, albumin levels in the treatment group were compared with normal control groups that were not given chlorpyrifos. So, by including the same age in the research inclusion criteria, getting the same type of food, and using the same environmental conditions, it is expected to reduce the possibility of bias in this study that can affect the study results. The results of this study can be the basis for further research, for example, on the effect of chlorpyrifos on drug pharmacokinetics, especially in terms of drug distribution bound to albumin.

CONCLUSION

This study concluded that the duration of low-dose chlorpyrifos exposure can cause changes in serum albumin levels in Wistar rats. Albumin levels in the chlorpyrifos administration group were significantly lower than those without chlorpyrifos. This result proves that the longer the organism is exposed to low-dose chlorpyrifos pesticides, the lower the organism's albumin levels.

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